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Validation of a UHPLC-ESI-MS/MS Method for Anthocyanidin Quantification in Potato Tubers

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Abstract

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The development of bioanalytical methods has become challenging due to sample complexity, requirements for method reliability, and speed of analysis with triple quadrupole LC-MS/MS used widely for the routine analysis of biological materials. The article presents the method development and validation results for pelargonidin and malvidin in potato tubers. The developed method uses a short C18 column, is able to measure all six common anthocyanidins, uses a binary mobile phase with acetonitrile and water both with added 1% formic acid, ESI ionisation in positive mode, 3-h hydrolysis with 2.7 M methanolic HCl at 90°C. For pelargonidin and malvidin, the method shows high recovery of 98–100%, intra-day repeatability of 6.7–17.9% (depending on the analyte and concentration level), uncertainty below 20%, and uses quadratic calibration.

Keywords: acid hydrolysis; method development; *Solanum tuberosum*

The development of bioanalytical methods has become more and more challenging over the past years due to the very demanding requirements in terms of method reliability, sensitivity, speed of analysis, and sample throughput. LC-MS/MS has established itself as a method of choice for the routine analysis of biological materials with triple quadrupole being the most widespread mass analyser in the routine LC-MS analysis (Novakova 2013). Method validation is a necessary process to demonstrate the ability of the developed method to provide quality, reliable, and trustworthy results in routine use (González *et al*. 2014; Karageorgou & Samanidou 2014).

Anthocyanins are a very complex class of molecules and comprise dozens of closely related forms (Andre *et al*. 2007). Therefore, the analysis of each particular form would be very expensive, time-consuming, and usually incomplete due to the lack of analytical standards. The analysis of anthocyanin aglycones (anthocyanidins) after suitable hydrolysis is cheaper and less time-consuming, and is able to provide enough specific information to the plant breeder or food scientist.

Since anthocyanidins are neither drugs, nor additives, nutrients, nor contaminants, no easy and straightforward implementation of the available validation guidelines (such as 2002/657/EC, FDA, AOAC) is possible. Also, anthocyanidin content varies greatly among potato cultivars and tubers; no certified reference material and isotopically labelled standards are commercially available. Since the common anthocyanidins possess very similar physical and chemical properties, the time-consuming validation procedure was carried out only for pelargonidin, which is the major component of red-fleshed potato tubers, and for malvidin, which is the major component of dark purple-fleshed potato tubers.

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Malvidin, petunidin, delphinidin, and peonidin are the most common anthocyanidins found in purple-fleshed potatoes, while pelargonidin is in red-fleshed potato tubers (Brown *et al*. 2008; Tierno *et al*. 2015). Anthocyanin content is routinely determined by a simple spectrophotometric method (Lachman *et al*. 2012). The aim of this paper is to present the data acquired during the development and validation of a fast routine quadrupole LC-MS/MS quantification method for pigmented potato tubers suitable for nutritional and breeding purposes.

MATERIAL AND METHODS

Plant material. Potato tubers (Table 1) came from the agricultural research locality at Uhříněves (Czech Republic). The analytical sample was prepared by freeze-drying (Lyovac GT2; Steris, Germany) thin wedges from 20 randomly selected tubers (with skin) for 7 days. The dry wedges were ground (IKA A11 basic; Germany) and stored refrigerated at 4°C. To collect validation data and monitor the method performance two in-house reference samples were created (separately for purple and red cultivars).

Chemicals. Analytical standards of pelargonidin chloride, cyanidin chloride, delphinidin chloride, malvidin chloride, petunidin-3-glucoside, and malvidin-3-glucoside were purchased from Extrasynthese (France); petunidin chloride and peonidin chloride from ChromaDex (USA). Methanol, acetonitrile (both HPLC grade), ethanol absolute, and hydrochloric acid (35%) were purchased from Lach-Ner (Czech Republic). Formic acid (HPLC grade) and alpha-amylase were obtained from Acros Organics (Belgium). If not mentioned otherwise, all chemicals were of, at least, GR grade or higher. Deionised HPLC grade water (≥ 18 MΩ) was prepared by Simplicity UV (EMD Millipore, Germany).

Sample preparation. 50 mg of freeze-dried sample powder was weighed into a 50-ml clear glass bottle (Shott Duran, Germany) and filled with 50 ml of hydrolysing medium (2.7 M HCl in methanol). The bottle was sonicated for 5 min and then placed into a pre-heated forced air oven (Venticell 111; BMT, Czech Republic) for 3 h at 90°C. Subsequently, the bottle was cooled down and its contents quantitatively transferred into a 100-ml volumetric flask (filled up with methanol). Further 1 : 10 (v/v) dilution of this solution in 1 M HCl in methanol was done. The sample was filtered through a 0.22 µm PTFE syringe filter and analysed. For calibration, separate pelargonidin and malvidin standard stock solutions (40 µg/ml) in 1 M HCl in methanol were prepared and stored refrigerated at 4°C. A combined standard solution of 2 µg/ml in 1 M HCl in methanol was used to create an 8-point calibration curve.

LC-MS/MS method. A 3200 QTRAP triple quadrupole mass spectrometer (AB Sciex, USA) with ESI source coupled with UHPLC (UltiMate 3000 RS, Thermo Fisher Scientific, USA) and nitrogen generator (Parker, USA) was used. Chromatographic conditions were as follows: total run time 7 min; Phenomenex Kinetex C18 column (2.1 × 30 mm, 1.7 µm particle size) kept at 25°C; a binary mobile phase consisting of (A) water with 1% formic acid (v/v) and (B) acetonitrile with 1% formic acid (v/v); gradient elution of 10% B in 0 min, 10% B in 1 min, increasing to 50% B in 5 min, decreasing to 10% B in 5.1 min and 10% B in 7 min; flow rate 350 µl/min.; injection volume 1 µl; autosampler temperature 4°C.

Statistics. All data were computed in Excel 2007 (Microsoft, USA), Statistica 12 (StatSoft Inc., USA), Analyst 1.4 (AB Sciex, Canada), and Chromeleon 6.8 (Thermo Fisher Scientific, USA). A two-way ANOVA, Tukey's test, and *t*-test on the significance level $\alpha = 0.05$ were applied.

RESULTS AND DISCUSSION

Method development. Optimised source and compound parameters are shown in Table 2. The lowest variability of results was achieved using formic acid and ion spray voltage (ISV) of +1500 which gave the highest signal intensity and signal stability (Table 3). Sample chromatograms are shown in Figure 1.

Table 1. List of potato cultivars

Potato group	Cultivars
Purple	Blaue de la Mancha[a], Blaue Anneliese[i], Blaue St. Galler[ai], Blue Congo[a], Blaue Elise[a], Boravaley[ai], Königsblau[ai], Salad Blue[ai], Valfi[ai], Violette[i], Vitelotte[ai]
Red	Herbie 26[ai], Highland Burgundy Red[ai], Königspurpur[ai], Rosalinde[ai], Rotte Emma[ai]

[a]cultivars used in method testing; [i]cultivars used to create an in-house reference sample

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Table 2. Ion source and compound parameters

Source parameters		Compound	Compound parameters							
			Q1	Q3	ion ratio	DP	EP	CEP	CE	CXP
			(Da)			(V)				
Curtain gas (AU)	20	pelargonidin	271.2	121.2	0.481	63.2	10.0	19.1	35.0	3.5
Collision gas (AU)	medium			141.3		63.2	10.0	19.1	50.0	3.5
Ion spray voltage (V)	1500	cyanidin	287.1	137.2	0.724	73.5	10.0	19.5	47.0	3.5
Temperature (°C)	700			213.0		79.6	10.0	19.5	42.0	3.5
Gas 1 (AU)	20	peonidin	301.1	201.1	0.605	62.3	10.0	19.9	51.0	3.5
Gas 2 (AU)	40			286.2		55.4	10.0	19.9	35.0	3.5
Interface heater	on	delphinidin	303.1	229.1	0.320	69.1	10.0	19.9	43.0	3.5
Electrode *x*-axis position (mm)	5			173.3		69.1	10.0	19.9	43.0	3.5
Electrode *y*-axis position (mm)	2	petunidin	317.1	245.1	0.561	56.8	10.0	20.3	51.0	3.5
				302.1		62.7	10.0	20.3	39.0	3.5
		malvidin	331.1	315.3	0.675	55.3	10.0	20.7	43.0	3.5
				287.1		53.9	10.0	20.7	45.0	3.5

AU – arbitrary units; Q1 – quadrupole 1; Q2 – quadrupole 2; DP – declustering potential; EP – entrance potential; CEP – collision cell entrance potential; CE – collision energy; CXP – collision cell exit potential

Based on the work of Xu *et al.* (2012), it was proved that hydrolysis at 90°C with 2.7 M HCl for 3 h is the most effective to release anthocyanidins from the potato matrix with recovery not being statistically different from 100% ($P > 0.983$).

The analyte stability in a stock solution (40 µg/ml, dissolved in 1 M HCl in methanol) stored at 4°C for 9 weeks was determined spectrophotometrically (Giusti *et al.* 1999) at 529 nm for pelargonidin (ε = 32 400) and 554 nm for malvidin (ε = 36 200). There was no statistical difference ($P > 0.979$) in the analyte concentration during the 10-week period either for pelargonidin or malvidin (Figure 2) and thus both stock solutions can be deemed as stable during that period if stored tightly sealed at 4°C. Two in-house reference materials (representing red-fleshed cultivars and purple-fleshed cultivars) were tested for stability. During the twelve-month period, the average value for malvidin dropped by 24.9% (CV 9.1%) and by 18.4% (CV 7.8%) for pelargonidin. Both values were statistically different from 100%, signifying a lesser long-term stability.

Method validation. For the purposes of validation, two in-house reference materials and three analysts were used and results were gathered in duplicates.

Table 3. Optimisation of liquid chromatography conditions

Mobile phase		Sample solvent for HPLC analysis	Analyte solutions	ISV (V)	Variability (%)		Average peak area (AU)	
A	B				PEL	MAL	PEL	MAL
1% FA in H_2O	1% FA in ACN	2.7 M HCl in MeOH	separate	4 500	47.29	60.16	73 230	87 210
		1 M HCl in MeOH			6.94	7.73	55 130	71 730
		5% FA in H_2O			22.38	54.02	34 040	24 790
5% FA in H_2O	5% FA in ACN	5% FA in H_2O	mixed	4 500	11.68	12.40	39 570	36 710
		2.7 M HCl in MeOH			11.62	17.35	4 694	15 780
		1 M HCl in MeOH			8.39	10.44	49 150	55 220
1% FA in H_2O	1% FA in ACN	2.7 M HCl in MeOH	mixed	1 500	12.21	7.31	38 840	72 220
		1 M HCl in MeOH			4.39	3.15	753 800	716 800
	MeOH	1 M HCl in MeOH			5.05	5.85	497 700	633 900

AU – arbitrary units; FA – formic acid; ACN – acetonitrile; MeOH – methanol; HCl – hydrochloric acid; ISV – ion spray voltage; PEL – pelargonidin; MAL – malvidin

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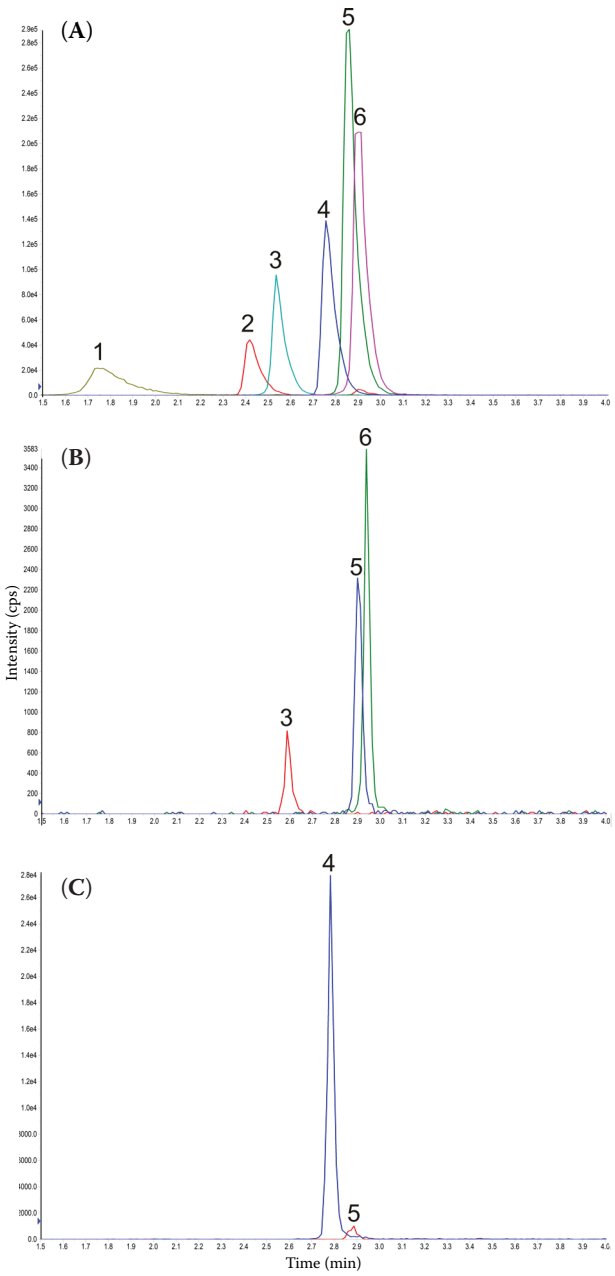

Figure 1. Chromatograms of potato samples in TIC mode showing the quantifier ion: (**A**) analytical standards, (**B**) purple potato, and (**C**) red potato

1 – delphinidin; 2 – cyanidin; 3 – petunidin; 4 – pelargonidin; 5 – peonidin; 6 – malvidin

In our study, the selection of one quantifier and one qualifier ion together with fragment ratio and comparison with retention times of external standards was deemed to be an adequate proof of selectivity and analyte identity.

Trueness (bias) of the method was investigated by spiking studies using pelargonidin-3-glucoside and malvidin-3-glucoside since anthocyanidins are predominantly found in glycosylated forms in potatoes (Burmeister *et al.* 2011) and expressed as recovery and matrix effects. Matrix effects were assessed by post-column infusion. Our experiments have proved that no ion enhancement/suppression occurs in the potato matrix for any of the anthocyanidins, which might be due to the sufficient matrix dilution after hydrolysis prior to injection.

To estimate precision and recovery, spiking experiments in 3 different matrices (yellow as a matrix blank, purple, and red potato) were used and measured during 8 weeks in duplicates. Recovery for pelargonidin was 97.68% (CV 5.82%) and for malvidin 100.35% (CV 7.92%). Statistically, neither value was found to be different from 100% ($P > 0.957$). There is no minimum established value for recovery, even though some pharmaceutical guidelines mention the range of 70–120% as acceptable.

Precision indicators (inter-day and intra-day) from the 8-week study are shown in Table 4. In real samples, the intra-day CV was for both analytes under 15% required e.g. by FDA. Inter-day reproducibility (also investigating different analysts) was higher, ranging from 19.5% to 29.1%. Calibration was assessed by twelve matrix matched calibration standards (2 ng/ml to 1 µg/ml) according to Rozet *et al.* (2011). Adequate linearity could be observed only in a very narrow range unsuitable for the routine analysis. Our results have shown that, in this range, a quadratic calibration curve (using a weighing factor 1/x to minimise overestimation of high concentration standards) fits best the data. The coefficient of determination (r^2) for pelargonidin was 0.9998, for malvidin 0.9999, and the accuracy for each calibration point ranged between 97.6 and 102.3% for both analytes. Using this calibration curve, the working range of the method is 75 µg/g DW to 13.4 mg/g

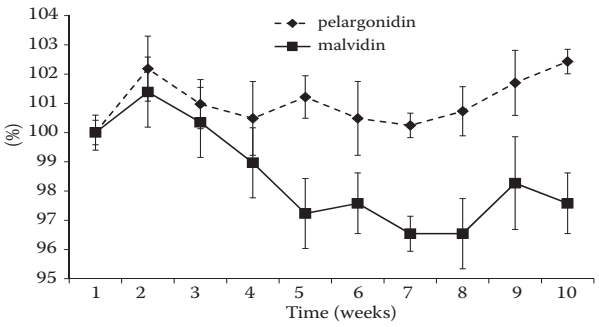

Figure 2. Stability of the analyte stock solution kept in 1 M HCl in methanol and measured in 0.1% HCl in ethanol (average of three replicates ± SD)

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Table 4. Method repeatability

Spiked with	Yellow		Red		Purple	
	PEL	MAL	unspiked	PEL	unspiked	MAL
Average value (µg/g)	288.5	330.9	1054.5	1320.4	1204.7	1524.0
SD	53.9	45.1	104.7	88.6	122.5	110.0
Intra-day repeatability (%)	17.8	13.5	10.0	6.7	10.4	7.2
Inter-day repeatability (%)	53.6	39.3	28.5	19.1	29.1	20.7

MAL – malvidin; PEL – pelargonidin

DW for pelargonidin and 55 µg/g DW to 11.4 mg/g DW for malvidin.

Limits of detection and quantitation (LOD, LOQ) were calculated according to Kruve *et al.* (2015). Pelargonidin LOQ corresponds to 75.03 µg/g DW (2.8 ng/ml) and malvidin LOQ to 54.74 µg/g DW (2.0 ng/ml). Since LOQ is defined as the lowest amount of the compound of interest in a sample that can be measured with an acceptable level of precision and accuracy (Bozovic & Kulasingam 2013), then the CV for pelargonidin and malvidin at LOQ level is 8.21 and 9.10%, respectively, which is in line with FDA requirements (CV ≤ 20%).

Uncertainty is usually expressed as a multiple of standard deviation. Using a factor of 2, there is about 95% probability that the true measurement result is within this interval (Rozet *et al.* 2011). Using a top-down approach and the results collected during the validation study, the uncertainty was calculated according to the AOAC guidelines for pelargonidin in red potatoes as being 19.24% and for malvidin in purple potatoes as being 19.7%.

Due to the very fact that there are only few validation parameters available for comparison either on anthocyanidins, potato matrix, or LC-MS/MS detection, it is very difficult to compare achieved results (also because researchers use different experimental designs and conditions for gathering validation parameters). Our validated method achieves lower LOQs compared to Shim *et al.* (2015) which were 0.48 mg/kg for pelargonidin and 0.91 mg/kg for malvidin, a much shorter chromatographic run compared to Nyman and Kumpulainen (2001) 25 min, and has similar recoveries as Xu *et al.* (2012) achieved for grape juice (99.9–102.8%). Compared to Shim *et al.* (2015), similar intra-day precision was achieved for red and purple potato yet with higher inter-day values which in our study design shows the effect (variability contribution) of three different analysts carrying out the same analytical procedure. Having validated the method and tested the method it is possible to conclude that the method we have developed for potato matrix is fit-for-purpose for measuring reliably anthocyanidins in freeze-dried pigmented potato tubers.

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